



ENVIRONMENTAL
HEALTH
PERSPECTIVES

<http://www.ehponline.org>

Carotid Intima-Media Thickness and Plasma Asymmetric Dimethylarginine in Mexican Children Exposed to Inorganic Arsenic

**Citlalli Osorio-Yáñez, Julio C. Ayllon-Vergara,
Guadalupe Aguilar-Madrid, Laura Arreola-Mendoza,
Erika Hernández-Castellanos, Angel Barrera-Hernández,
Andrea De Vizcaya-Ruiz and Luz M. Del Razo**

<http://dx.doi.org/10.1289/ehp.1205994>

Online 11 June 2013

Carotid Intima-Media Thickness and Plasma Asymmetric Dimethylarginine in Mexican Children Exposed to Inorganic Arsenic

Citlalli Osorio-Yáñez¹, Julio C. Ayllon-Vergara², Guadalupe Aguilar-Madrid³, Laura Arreola-Mendoza⁴, Erika Hernández-Castellanos¹, Angel Barrera-Hernández¹, Andrea De Vizcaya-Ruiz¹, and Luz M. Del Razo¹

¹Departamento de Toxicología, Centro de Investigación y de Estudios Avanzados del IPN, México D.F. México

²Hospital Español, México D.F., México

³Unidad de Investigación y Salud en el Trabajo, Instituto Mexicano del Seguro Social, México D.F., México

⁴Departamento de Biociencias e Ingeniería CIIEMAD-IPN, México D.F., México

Corresponding Author:

Luz María Del Razo PhD

Departamento de Toxicología

Centro de Investigación y de Estudios Avanzados del IPN, CINVESTAV-IPN

Av. Instituto Politécnico Nacional 2508, México D.F. 07360

Phone (555)747-3306; Fax (555) 574-73395

E-mail: ldelrazo@cinvestav.mx

Key Words: adhesion molecules; asymmetric dimethyl arginine; carotid intima media thickness; children; inorganic arsenic exposure; subclinic atherosclerosis.

Short running title: cIMT, ADMA, and As

Disclosure: The authors declare that they do not have any competing financial interests to disclose.

Acknowledgments: The authors thank study children and their families in Zimapan Hidalgo, who generously contributed to this project and also thank Luz del Carmen Sanchez-Peña, Eva Margarita Melgar Paniagua and Marco Antonio Sánchez Guerra (Departamento de Toxicología, CINVESTAV) and the staff of Jurisdicción Sanitaria 5 de Zimapan, Hidalgo, for their extraordinary efforts with subject recruitment, interviews, medical exams, and sample collection.

Abbreviations:

ADMA	asymmetric dimethylarginine
BMI	body mass index
cIMT	carotid intima media thickness
CINVESTAV-IPN	Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional
DMAs	dimethylarsenic
HDL	high density lipoprotein
HG-CT-AAS	hydride generation-cryotrapping-atomic absorption spectrometry
iAs	inorganic arsenic
LDL	low density lipoprotein
MAAs	methyl-As
NO	nitric oxide
NOS	nitric oxide synthase
NOx	nitrate and nitrite
ROS	reactive oxidative specie
ICAM-1	soluble intercellular adhesion molecule-1
sVCAM-1	soluble vascular cell adhesion molecule-1
tAs	total arsenic
VSMC	vascular smooth muscle cells
VLDL	very low density lipoprotein

Abstract

Background: Arsenic exposure is a risk factor for atherosclerosis in adults, but there is little information on arsenic and early risk biomarkers for atherosclerosis in children. Carotid intima-media thickness (cIMT) is an indicator of subclinical atherosclerotic burden that has been associated with plasma asymmetric dimethylarginine (ADMA), a predictor of cardiovascular disease risk.

Objectives: The aim of this study was to investigate associations of arsenic exposure with cIMT, ADMA, and endothelial adhesion molecules (soluble intercellular cell adhesion molecule-1, sICAM-1; soluble vascular cell adhesion molecule-1, sVCAM-1) in children who had been exposed to environmental inorganic arsenic (iAs).

Methods: A cross-sectional study was conducted in 199 children 3 to 14 years of age who were residents of Zimapan, México. cIMT was evaluated using ultrasonography; plasma lipid profiles were evaluated by standard methods, ADMA, sICAM-1, and sVCAM-1 were analyzed by ELISA; and the concentrations of total speciated arsenic (tAs) in urine were measured using HG-CT-AAS.

Results: In the multiple linear regression model for cIMT, tAs categories were positive associated with cIMT increase, the estimated cIMT diameter were greater in 35-70 and >70ng/mL groups (0.035 mm and 0.058 mm per 1 ng/mL increase in urinary tAs, respectively), compared to the <35 ng/mL. In addition to tAs level, plasma ADMA was significant predictor of cIMT. In adjusted regression model for plasma ADMA, cIMT, %iAs, and plasma sVCAM-1 were significant predictors of ADMA levels (e.g., 0.419 $\mu\text{mol/L}$ increase in ADMA per 1mm increase in cIMT).

Conclusions Arsenic exposure and plasma ADMA levels were positively associated with cIMT in a population of Mexican children with environmental arsenic exposure through drinking water.

INTRODUCTION

Inorganic arsenic (iAs) is naturally occurring and ubiquitous in the environment. In most populations, drinking water is the main source of human exposure. Long-term iAs exposure has been associated with coronary disease, stroke, ischemic heart disease, hypertension, and carotid atherosclerosis in adults (States et al. 2009). Epidemiological studies conducted in Taiwan have demonstrated that long-term iAs exposure is significantly associated with carotid atherosclerosis in adults, with a positive dose-response relationship after adjustment for other cardiovascular risk factors, which suggests that iAs exposure may be an independent risk factor for atherosclerosis (Wang et al. 2002, 2007). However, to our knowledge, there have been no epidemiologic studies on the role of iAs exposure in atherosclerosis initiation or progression in pediatric populations. Atherosclerosis is a multistage disease that can initiate in childhood and remain subclinical until adulthood, when it becomes clinically manifest. Atherosclerosis is a multistage disease that can initiate in childhood and remain subclinical until adulthood, when it becomes clinically manifest. Carotid artery wall intima-media thickness (cIMT) is a widely accepted indicator of subclinical atherosclerotic burden, and its determination could be useful in identifying young adults at risk for premature coronary atherosclerosis (Slyper 2004). Epidemiological evidence suggests a close association between cIMT and plasma asymmetric dimethylarginine (ADMA) concentrations (Ayer et al. 2009). ADMA at baseline predicted subsequent cardiovascular disease in a 22-year follow study of adult women (Leong et al. 2008). Clinical conditions with elevated plasma ADMA concentrations in children including hypertension, hypercholesterolemia, chronic kidney disease, and diabetes mellitus (Tain and Huang 2011). However, there is little toxicological evidence regarding ADMA. The association of ADMA with adverse clinical events could be related to the attenuation of the vascular protective effects of nitric oxide (NO). In animal

models, local inhibition of NO, accelerates early neointima formation (Cayatte et al. 1994). Moreover, Nanayakkara and coworkers found a positively association between ADMA and soluble vascular adhesion molecule-1 (sVCAM-1), a molecule expressed in activated endothelial cells, in patients with mild to moderate renal failure (Nanayakkara et al. 2005).

The aim of the present study was to investigate the association between iAs exposure and cIMT, plasma ADMA, and endothelial adhesion molecules in a pediatric population exposed to environmental iAs.

MATERIALS AND METHODS

Study Participants. A cross-sectional study was conducted in 199 children (3-14 years old) who were residents of the Zimapan region in Mexico. This study was approved by the Institutional Review Board of CINVESTAV-IPN. In this area, high concentrations of naturally occurring iAs are frequently found in the bedrock and, as a consequence, in underground and surface waters (Armienta and Rodriguez, 1996). The children were recruited from two local schools, and were residents of five area towns (Calvario, Llano Norte, Aguacatal, Muhi, and Downtown). At the time of evaluation, arsenic levels measured in the drinking water of these towns ranged from 3 to 135 ng As/mL. Before enrollment in the study, the parents read and signed a written informed consent form. Parents were interviewed by trained interviewers on general characteristics, with an emphasis on the source of drinking water, marine food consumption, second hand smoking exposure, and detailed residential information including if the mother lived in Zimapan area during pregnancy (yes/no), child allergies, child surgery interventions, medication, and child medical history. Only children with a minimum of one year of residency in the Zimapan region were eligible to participate. Children with diabetes or cardiovascular disease were excluded.

Child Exam and Sample Collection. Children were examined using carotid ultrasonography by an expert cardiologist who was blinded to the study design and participants' clinical data. The children were examined using a cardiovascular ultrasound system (Vivid i ®, General Electric, Milwaukee, WI, USA) equipped with a 14-MHz linear transducer (Milwaukee, WI, USA) following a standardized protocol using B-mode ultrasound with the child in a supine position with the head turned slightly to the left and right (Pignoli et al.1986). cIMT was calculated based on automatic contour detection of the intima and media layers in a user-defined search region along the vessel wall. Multiple cIMT measurements were made between pairs of intima and adventitia points along the posterior wall of the vessel. The following parameters were calculated: average cIMT (cIMTmean), maximum cIMT (cIMTmax), and minimum cIMT (cIMTmin).

We measured body weight and height using standard protocols. We calculated the body mass index (BMI) using the formula weight (kg)/height (m²). The BMI z-score was calculated and BMI was categorized based on guidelines of the Center of Disease Control and Prevention (<http://www.cdc.gov/>). Each participant provided a first void urine sample. Urinalysis was performed immediately, and samples were stored at -20°C at the local health clinic, until they were transported with cooling blocks to Mexico City for further analysis. A sample of approximately 12 h fasting venous blood was collected. Plasma was prepared from blood samples by centrifugation at 4°C and stored at -80°C.

Analysis of As in Water and Urine. Arsenic analysis included the analysis of sodium arsenite (NaAs^{III}O₂) and arsenic acid disodium salt (Na₂HAs^VO₄). Both of these chemicals (>99% pure) and dimethylarsinic acid [DMAs^V; as (CH₃)₂As^VO(OH); 99% pure] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methylarsonic acid (MAs^V) disodium salt

($\text{CH}_3\text{As}^{\text{V}}\text{O}(\text{ONa})_2$; 99% pure) was obtained from Ventron (Danvers, MA, USA). Working standards of these arsenicals, which contained 1 μg of As/mL, were prepared daily from stock solutions. Sodium borohydride (NaBH_4) and L-cysteine hydrochloride were obtained from EM Science (Gibbstown, NJ, USA). Ultrapure Tris-hydrochloride monohydrate was purchased from Sigma, and Tris-hydrochloride was purchased from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals used were at least analytical grade. The concentrations of total arsenic (tAs) in drinking water were determined by HG-atomic fluorescence spectrometry (HG-AFS), as previously described (Le and Ma 1998). Trace elements in standard water reference material (SRM 1643e, National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA) containing 60.4 ± 0.7 ng As/mL were used for quality control. HG-atomic absorption spectrometry (AAS) with a cryotrap (CT) for the capture and separation of hydrides was used for the analysis of iAs and its metabolites in urine (Hernández-Zavala et al. 2008). We used standard reference material (SRM 2669) from NIST for quality control in measurements of arsenic species in frozen human urine. We used NIST 2669 level I and level II to validate the analysis of arsenic species at low and elevated concentrations in the urine matrix, respectively. The low tAs concentration urine sample at NIST 2669 level I had a reference value of 9.22 ± 0.32 ng As/mL, and the reference value was 43.67 ± 0.63 ng As/mL for the high tAs concentration at NIST 2669 level II. Replicate analyses of SRM 2669 showed values with less than 10% coefficient of variation of reference values for the high and low standards. The sum of the concentrations of iAs, MAs and DMAs in urine was reported as tAs.

Plasma Analyses. The concentrations of glucose, total cholesterol, triglycerides and high-density lipoprotein (HDL) were measured in plasma by the end-point enzymatic method using fully automatic biochemistry analyzer (SYNCHRON LX 20, Beckman Coulter, Mexico) and

values of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) were calculated using the Friedewald formula (Friedewald et al. 1972). The atherogenic index was calculated for each child as total cholesterol/HDL. The hematocrit, hemoglobin and leucocytes were determined using a standard method. A second plasma aliquot was stored at -70°C and analyzed after 1 thaw cycle for ADMA using an enzyme-linked immunosorbent assay (ADMA human ELISA Kit, Immundiagnostik AG, Lörrach, Germany). ADMA was assessed in duplicate (intra-assay CV <15%), and the average was recorded as the ADMA level. Two controls were included in the kit assay to control for quality. The mean and range was 0.25 (0.19-0.32) $\mu\text{mol/L}$ for control level I and 0.76 (0.57-0.95) $\mu\text{mol/L}$ for level II. In each assay the values of the controls were in the range established by the manufacturer. In addition to ADMA quantification, adhesion molecules, such as sVCAM-1 and soluble intercellular adhesion molecule-1 (sICAM-1) were analyzed using enzyme-linked immunosorbent assays (Invitrogen, California, USA).

Evaluation of iAs Exposure and Metabolism. The concentration of tAs in the urine was used to estimate individual exposure to iAs. The proportion of arsenic in each species (%iAs,%MAs, and %DMAs) was calculated by dividing the concentration of arsenic in each species by the tAs in urine.

Statistical Analyses. Exploratory analyses were performed to assess data quality and consistency and the distribution of the variables of interest. Continuous variables presented non-normal distribution except ADMA and %MAs, which were normally distributed. All continuous variables are described as geometric mean (GM) and range and also the mean \pm the standard deviation are reported. Frequencies or percentages are reported for categorical variables. Simple linear regression models were used to estimate associations of ADMA and cIMT, with potential confounders (age, gender, body mass index or z-score, atherogenic index, lipid serum profile,

adhesion molecules, and fasting plasma glucose) and with urine tAs and arsenic species. The effect of *in utero* exposure was also evaluated by simple linear regression analyses on the two outcomes of interest. In addition to evaluating urine tAs as a continuous variable, we stratified exposure into three categories: <35 ng/mL [where 35 ng/mL represents the Biological Exposure Index (BEI) or permissible limit for occupational As exposure (ACGIH, 2004)], 35-70 ng/mL, and >70 ng/mL (2 times the BEI value). Additionally, a Wilcoxon type test for trend was performed to evaluate cIMT increase across arsenic categories (Figure 1). Pearson's correlation coefficient (r_p) for plasma ADMA associations and Spearman's correlation coefficient (r_s) for plasma sVCAM-1 and sICAM-1, were performed among the main exposure-outcome association. Multivariable linear regression analyses of associations with ADMA or cIMT were adjusted for potential confounding variables related to outcome, based on Wald tests with a p-value of <0.20, or if their inclusion improve the model fit (based on the change in 10% of R^2 value). The adjusted models also included age, which did not fulfill this statistical criterion but was considered to be biologically important. In the case of cIMT, the explanatory variable in the linear regression model was modeled as an untransformed continuous variable or as square transformed continuous variable. As inferences based on square transformed cIMT were comparable (data not shown), results are reported for cIMT as an untransformed variable for easier interpretation. Analysis for validation of the multiple regression with robust weight function were performed for cIMTmin analyses (Davidson and McKinnon 1993). While validation of the multiple regression ADMA model was performed using studentized residuals. Model predictions were graphed against standardized residuals to assess heteroscedasticity (Montgomery and Peck, 1992). P-values <0.05 were considered statistically significant. All statistical analyses were performed using Stata version 10 (StataCorp, College Station, TX).

Results

Characteristics of the study population. Of the initial 199 child participants, three had no urine or blood samples, one was excluded due to an atrial septal defect, and forty-seven children had no ultrasonography. Most of the children (70%) were < 6 years old (age range 3 to 14 years) (Table 1). In total, 79% of children had urinary tAs values higher than the BEI of 35 ng/mL, and only 21% showed levels less than this reference value. Using standard international BMI age- and gender-specific cut-off points, 66% were classified as normal weight, 7% underweight, 18% overweight, and 9% obese (Table 1). There were no significant differences between boys and girls in the parameters studied (data not shown), with the exception of glucose (86.11 in boys vs. 80.03 mg/dL in girls; $p=0.0001$), HDL (53.85 vs. 50.7 mg/dL; $p=0.0327$), hemoglobin (13.76 vs. 14.00%; $p=0.025$), and sICAM-1 (0.59 vs. 0.64 $\mu\text{g/mL}$; $p=0.0142$).

Only 22% of the population reported any seafood consumption, and only 16% were exposed to second hand smoke. Neither seafood consumption nor second hand smoke were significant predictors of cIMT or ADMA (data not shown).

In unadjusted models, triglycerides, VLDL, sICAM-1 and sVCAM-1 levels were significant predictors of plasma ADMA concentration, and age was a marginally significant predictor ($p = 0.06$) (Table 2). The association among plasma ADMA and urinary tAs, MAs, and DMAs concentration had a p -value less than 0.1. Gender, BMI z-score, and BMI categories did not predict plasma ADMA. Atherogenic index and BMI z-score were significant predictors of cIMTmin, and overweight and sICAM-1 were marginal predictors, while age and gender were not associated with cIMT (Table 2). In addition, cIMTmin diameter was positively associated with both unstratified and stratified urinary tAs (with a monotonic increase in geometric mean

cIMT with increasing categorical exposure). Compared with the lowest exposure group, estimated values of cIMT were 0.033 mm and 0.054 mm higher among those with tAs 35-70 and >70ng/mL, respectively (Table 2).

Association of cIMT with iAs Exposure. cIMTmin was significantly associated with urine tAs based on simple linear regression (Figure 1) and after adjustment for atherogenic factor, BMI z-score, age, and plasma ADMA (Table 3). In contrast, tAs exposure was not correlated with plasma lipids or BMI ($p>0.05$; data not shown). The multivariable regression model explained 18% of the variability in cIMTmin diameter, with the strongest associations estimated for plasma ADMA concentration (0.068 mm increase, 95% CI: 0.0117, 0.124 for a 1- $\mu\text{mol/L}$ increase in ADMA) and tAs >70 ng/mL (0.058 mm increase, 95% CI: 0.0198, 0.095 compared with tAs <35 ng/mL) (Table 3).

Associations of Cardiovascular Biomarkers with iAs Exposure and Metabolism. Plasma ADMA, sICAM-1, and sVCAM-1 were highly correlated ($p<0.05$), and ADMA and sICAM biomarkers were also correlated with triglycerides and VLDL (Supplemental Material, Table S1). BMI, glucose, total cholesterol, HDL, LDL and the atherogenic index were not significantly correlated with cardiovascular biomarkers (data not shown). ADMA was weakly correlated with urinary tAs ($r_p=0.122$; $p=0.092$), MAs ($r_p=0.121$; $p=0.093$), and DMAs ($r_p=0.128$; $p=0.076$). sVCAM-1 was significantly correlated with age ($r_s=-0.17$; $p=0.024$).

In multivariable regression analysis, iAs%, sVCAM-1, and cIMTmin were significantly associated with plasma ADMA (Table 4, Figure 2). The model explained 15% of the variability in plasma ADMA, with the strongest predictors being cIMTmin diameter (0.419 $\mu\text{mol/L}$ increase in ADMA per 1-mm cIMTmin), age (0.0314 $\mu\text{mol/L}$ decrease per year), and iAs% (0.0147

μmol/L increase per 1-unit iAs%). Age was a stronger predictor of ADMA than iAs%. DMAs% and triglycerides were also significant predictors of ADMA. Finally values of ADMA or cIMT diameter were not significant associated with *in utero* arsenic exposure ($p>0.05$; data not shown).

Discussion

Over recent decades, exposures to iAs in central Mexico and potential effects on human health have given rise to much concern (Del Razo et al. 2011). In our study of a pediatric population, we found that cIMT and plasma ADMA were increased in association As levels in urine.

Previous studies have reported that cIMT is increased in children at high risk for cardiovascular disease due to familial hypercholesterolemia, type I diabetes, offspring of parents with premature myocardial infarction, elevated levels of ultrasensitive C-reactive protein compared with other children (Jarvisalo et al. 2001; Slyper 2004). Epidemiological studies have also reported associations between long-term iAs exposure and outcomes related to atherosclerosis in adults, including ischemic heart disease, cerebrovascular disease, and peripheral vascular disease (Simeonova and Luster 2004). To our knowledge, ours is the first epidemiologic study to report an association between iAs exposure and cIMT in children. Specifically, we estimated a significant association between urinary tAs (35-70 ng/mL and >70 ng/mL) and cIMT_{min} in children, 70% of whom were less than six years old. In present study cIMT_{min} was significant associated with ADMA and urinary tAs. This suggests that the minimum cIMT diameter increase could represent the earliest morphological changes related to arsenic exposure and/or ADMA increase.

Few studies have focused on early cardiovascular effects of iAs exposure in children. One study based on autopsy findings for five children from an As-endemic area of Chile reported intimal thickening in the small- and medium-sized arteries; this similar vascular lesion was found in heart, stomach, intestines and mesentery (Rosenberg 1974). In other autopsy studies the earliest abnormalities observed in children 2-15 years of age with atherosclerotic risk factors were fatty streaks and fibrous plaques (Berenson et al. 1998; Newman et al. 1991). Endothelial dysfunction may be the initial phenomenon in subclinical atherosclerosis that precedes thickening in the vascular wall (Kallio et al. 2010). An increase in cIMT could be a consequence of effects of iAs on endothelial dysfunction, foam cell formation stimulation, reactive oxidative stress (ROS) production, proinflammatory chemokines and cytokines, inflammation, vascular smooth muscle cell (VSMC) proliferation, endothelial cell proliferation, platelet aggregation, and decreased fibrinolytic activity (Balakumar and Kaur 2009; Simeonova and Luster 2004; Wang et al. 2002).

In multivariable regression analyses, urine tAs, plasma ADMA, and the atherogenic index were significant predictors of increased cIMT. But, lipid serum profile was not correlated with tAs in our pediatric study population (data not shown), as one report based on adults in Taiwan with exposures to high levels of arsenic and ischemic heart disease (Hsueh et al. 1998). Although lipids in serum are intimately related with atherosclerosis, our results suggest that early iAs-mediated effects on atherogenesis may be independent of the lipid serum profile. Median concentrations of plasma total cholesterol, HDL, VLDL, LDL, and triglycerides were consistent with levels recommended for the primary prevention of atherosclerosis beginning in childhood (Kavey et al. 2003) and were within the normal range reported previously for Mexican children (Perichart-Perera et al. 2007).

Although we did not find association between *in utero* arsenic exposure on cIMT diameter or ADMA level, we cannot rule out a contribution of *in utero* arsenic exposure to the association between urine arsenic in childhood and cIMT because most of the mothers lived in the area during pregnancy (83%). Srivastava and coworkers hypothesized that accelerated development of aortic lesions and vasorelaxation defects observed in Apo E^{-/-} mice exposed to arsenic *in utero* was due to an arsenic-mediated reduction in NO availability (Srivastava et al. 2007). Our findings suggest that the association between urinary tAs and cIMT_{min} in our pediatric study population could have been mediated by an effect of iAs on ADMA, which is an endogenous inhibitor of NO.

Adhesion molecules and ADMA have been studied in children at risk of cardiovascular disease due to hypertension, obesity, and a family history of cardiovascular disease (Ayer et al. 2009; Goonasekera et al. 2000). Adhesion molecules, such as ICAM-1 and VCAM-1, have been used as early biomarkers of atherosclerosis due to their participation in the initial step of the disease, in which they promote the translocation of monocytes and leucocytes to the arterial endothelium with subsequent migration to the sub-endothelial space, initiating the atherosclerosis process (Chen et al. 2007; Glowinska et al. 2003). Although ICAM-1 may be a less specific marker than VCAM-1, which is primarily expressed by activated endothelial cells and muscle cells in atherosclerotic plaques (Blake and Ridker 2002), it is more predictive of cardiovascular disease in apparently healthy subjects (de Lemos et al. 2000). sVCAM-1 and sICAM-1 have been correlated with iAs exposure in adults (Chen et al. 2007). In contrast, we did not find significant associations between both adhesion molecules and any arsenicals in the urine. Prospective cohort studies have reported that the plasma concentration of sICAM-1 is elevated many years prior to an initial myocardial infarction (Hwang et al, 1997; Ridker et al, 1998) but sICAM-1 has also

been reported to be elevated in children with acute otitis media caused by bacterial infections (Liu et al. 2010). In contrast with the adhesion molecules, ADMA was associated with cIMT and with the relative proportion of iAs in our study population. In adults with peripheral arterial occlusive disease there is a progressive reduction in urinary nitrate and cGMP rates (markers of NO formation), which may be caused in part by accumulation of ADMA (Böger et al.1997). ADMA plasma concentrations in patients with end renal stage disease were higher in hemodialysis patients with manifested atherosclerosis disease compared with hemodialysis patients without atherosclerosis disease (Kielstein et al. 1999). Nevertheless, additional research is needed to establish the utility of ADMA as a biomarker of environmentally-mediated cardiovascular disease.

Urine MAs% was associated with carotid atherosclerosis in a previous case-control study of adults (Wu et al. 2006). MAs% was not a significant predictor of cIMT in our study population, but DMAs% was weakly associated with plasma ADMA concentration in the multivariable model ($p = 0.086$). This result could be explained by methylation capacity differences between children and adults. Children in Bangladesh were reported to have lower urinary MAs% and higher DMAs% than adults, suggesting that the second step in arsenic methylation may be more active in children (Chowdhury et al.2003). We found a positive and significant association between urine iAs% and plasma ADMA in the multivariable regression analysis (Table 4). A recent study of surgical samples from 3 coronary heart disease patients that lived in arsenic-area in Chile reported that iAs was the predominant arsenic specie in cardiovascular tissue, whereas DMAs and MAs levels in the same samples were relatively low or undetectable (e.g. iAs concentration in auricle was 49.2 $\mu\text{g/g}$, whereas MAs and DMAs were undetectable (Roman et al. 2011). In Apo E^{-/-} mice, an animal model of atherosclerosis, atheroma formation in arsenic

exposed mice was accompanied by increasing levels of iAs in the vessel wall (Simeonova et al. 2003).

The relative proportion of urine iAs in was significantly associated with plasma ADMA in our pediatric study population, in which many potential confounding factors, such as smoking, diabetes, and sedentary lifestyle, were absent. This suggests that ADMA might affect the arterial wall early in life, rather than being a biomarker of age-related vascular degeneration only, and that ADMA might play a role in early iAs-mediated atherosclerotic effects. ADMA is an endogenous inhibitor of nitric oxide synthase (NOS) that is derived from the proteolysis of proteins containing methylated arginine residues. NO has been characterized as the “endogenous antiatherosclerotic molecule” due to its antithrombotic, antioxidant, and vasodilatation properties, among others (Böger 2003). Therefore, any condition that reduces NO may promote atherosclerosis. A study of adults in Inner Mongolia, China exposed to high levels of arsenic in well water reported a negative association between iAs exposure and stable plasma metabolites of NO, including nitrite and nitrate (NO_x) (Pi et al. 2000). Interestingly, the most robust correlation with NO_x depletion was iAs% in blood ($p < 0.001$). Although we did not measure As in blood, multivariable regression analysis showed that iAs% in the urine was associated with plasma ADMA. Although several mechanisms underlying iAs exposure-mediated NO depletion have been proposed, the association is not completely understood (Kumagai and Pi 2004). We believe that our findings suggest a new pathway in which iAs exposure could decrease NO levels and promote atherosclerotic disease. However, evidence of NO-independent effects of ADMA on microvascular lesions in NOS-knock out and wild-type mice has also been reported (Suda et al. 2004).

To our knowledge, ours is the first epidemiologic study to implicate ADMA in subclinical atherosclerosis due to iAs exposure. In an experimental study of myelin alteration in rats exposed to iAs via drinking water (36 µg/mL), plasma ADMA was significantly increased 4 months after treatment compared with the control group (9.7 ± 0.6 vs. 3.4 ± 0.6 ; nmol/mL; Zarazua et al. 2010).

We cannot rule out the possibility of bias due to confounding by factors such as a family history of stroke, cardiovascular disease or diabetes, and the temporal relations between exposure and the outcomes that we evaluated cannot be established due to the cross-sectional nature of our analysis. However, additional studies are warranted given our findings of an association of iAs with cIMT and plasma ADMA in children.

References

- American Conference of Governmental Industrial Hygienists, 2004. Arsenic and soluble Inorganic compounds: BEI®. ACGIH. 8th. Edition Documentation, Cincinnati, Ohio. <http://www.acgih.org/Store/ProductDetail.cfm?id=1368>
- Armienta MA, Rodriguez R, Aguayo A, Cenicerros N, Villaseñor G, Cruz O. 1997. Arsenic contamination of groundwater at Zimapan, Mexico. *Hydrogeol J* 5:39-46.
- Ayer JG, Harmer JA, Nakhla S, Xuan W, Ng MK, Raitakari OT, et al. 2009. HDL-cholesterol, blood pressure, and asymmetric dimethylarginine are significantly associated with arterial wall thickness in children. *Arterioscler Thromb Vasc Biol* 29:943-949.
- Balakumar P, Kaur J. 2009. Arsenic exposure and cardiovascular disorders: an overview. *Cardiovasc Toxicol* 9:169-176.
- Berenson GS, Srinivasan SR, Bao W, Newman WP, 3rd, Tracy RE, Wattigney WA. 1998. Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study. *N Engl J Med* 338:1650-1656.
- Blake GJ, Ridker PM. 2002. Inflammatory bio-markers and cardiovascular risk prediction. *J Intern Med* 252:283-294.
- Böger RH, Bode-Boger SM, Thiele W, Junker W, Alexander K, Frolich JC. 1997. Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. *Circulation* 95:2068-2074.
- Böger RH. 2003. Association of asymmetric dimethylarginine and endothelial dysfunction. *Clin Chem Lab Med* 41:1467-1472.
- Cayatte AJ, Palacino JJ, Horten K, Cohen RA. 1994. Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. *Arterioscler Thromb* 14:753-759.
- CDC (Centers for Disease Control and Prevention). 2000. CDC growth charts. Atlanta GA, <http://www.cdc.gov/growthcharts> [accessed 26 June 2011].
- Chen Y, Santella RM, Kibriya MG, Wang Q, Kappil M, Verret WJ, et al. 2007. Association between arsenic exposure from drinking water and plasma levels of soluble cell adhesion molecules. *Environ Health Perspect* 115:1415-1420.

- Chowdhury UK, Rahman MM, Sengupta MK, Lodh D, Chanda CR, Roy S, et al. 2003. Pattern of excretion of arsenic compounds [arsenite, arsenate, MMA(V), DMA(V)] in urine of children compared to adults from an arsenic exposed area in Bangladesh. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 38:87-113.
- de Lemos JA, Hennekens CH, Ridker PM. 2000. Plasma concentration of soluble vascular cell adhesion molecule-1 and subsequent cardiovascular risk. *J Am Coll Cardiol* 36:423-426.
- Davidson R, McKinnon JG. 1993. *Estimation and Inference in Econometrics*, Oxford University Press.
- Del Razo LM, García-Vargas GG, Valenzuela OL, Castellanos EH, Sánchez-Peña LC, Currier JM, et al, 2011. Exposure to arsenic in drinking water is associated with increased prevalence of diabetes: a cross-sectional study in the Zimapán and Lagunera regions in Mexico. *Environ Health* 24:10:73. .
- Friedewald WT, Levy RI, Fredrickson DS. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical chemistry* 18: 499-502.
- Glowinska B, Urban M, Peczynska J, Szczepanska-Kostro J. 2003. [Selected adhesion molecules: sICAM-1 and sVCAM-1 as markers of endothelial dysfunction in diabetic children and adolescence]. *Pol Merkur Lekarski* 14: 205-209.
- Goonasekera CD, Shah V, Rees DD, Dillon MJ. 2000. Vascular endothelial cell activation associated with increased plasma asymmetric dimethyl arginine in children and young adults with hypertension: a basis for atheroma? *Blood Press* 9:16-21.
- Hernández-Zavala A, Matoušek T, Drobná Z, Paul DS, Walton F, Adair BM, et al. 2008. Speciation of arsenic in biological matrices by automated hydride generation-cryotrapping-atomic absorption spectrometry with multiple microflame quartz tube atomizer (multiatomizer). *J Anal At Spectrom* 23:342 -351.
- Hsueh YM, Wu WL, Huang YL, Chiou HY, Tseng CH, Chen CJ. 1998. Low serum carotene level and increased risk of ischemic heart disease related to long-term arsenic exposure. *Atherosclerosis* 141:249-257.

- Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM Jr et al. 1997. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. *Circulation* 96:4219-4225.
- Jarvisalo MJ, Jartti L, Nanto-Salonen K, Irjala K, Ronnema T, Hartiala JJ, et al. 2001. Increased aortic intima-media thickness: a marker of preclinical atherosclerosis in high-risk children. *Circulation* 104:2943-2947.
- Kallio K, Jokinen E, Saarinen M, Hamalainen M, Volanen I, Kaitosaari T, et al. 2010. Arterial intima-media thickness, endothelial function, and apolipoproteins in adolescents frequently exposed to tobacco smoke. *Circ Cardiovasc Qual Outcomes* 3:196-203.
- Kavey RE, Daniels SR, Lauer RM, Atkins DL, Hayman LL, Taubert K. 2003. American Heart Association guidelines for primary prevention of atherosclerotic cardiovascular disease beginning in childhood. *Circulation* 107:1562-1566.
- Kielstein JT, Boger RH, Bode-Boger SM, Schaffer J, Barbey M, Koch KM, et al. 1999. Asymmetric dimethylarginine plasma concentrations differ in patients with end-stage renal disease: relationship to treatment method and atherosclerotic disease. *J Am Soc Nephrol* 10:594-600.
- Kumagai Y, Pi J. 2004. Molecular basis for arsenic-induced alteration in nitric oxide production and oxidative stress: implication of endothelial dysfunction. *Toxicol Appl Pharmacol* 198:450-457.
- Le XC, Ma M. 1998. Short-column liquid chromatography with hydride generation atomic fluorescence detection for the speciation of arsenic. *Anal Chem* 70:1926-1933.
- Leong T, Zylberstein D, Graham I, Lissner L, Ward D, Fogarty J, et al. 2008. Asymmetric dimethylarginine independently predicts fatal and nonfatal myocardial infarction and stroke in women: 24-year follow-up of the population study of women in Gothenburg. *Arterioscler Thromb Vasc Biol* 28:961-967.
- Liu K, Casey J, Pichichero M. 2010. Serum intercellular adhesion molecule 1 variations in young children with acute otitis media. *Clin Vaccine Immunol* 17:1909-1916.
- Montgomery D C and Peck E A. (1992), 2nd Edition, Introduction to linear regression analysis, A Wiley-Interscience, USA / Canada.

- Nanayakkara PW, Teerlink T, Stehouwer CD, Allajar D, Spijkerman A, Schalkwijk C, et al. 2005. Plasma asymmetric dimethylarginine (ADMA) concentration is independently associated with carotid intima-media thickness and plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) concentration in patients with mild-to-moderate renal failure. *Kidney international* 68: 2230-2236.
- Newman WP, 3rd, Wattigney W, Berenson GS. 1991. Autopsy studies in United States children and adolescents. Relationship of risk factors to atherosclerotic lesions. *Ann N Y Acad Sci* 623:16-25.
- Perichart-Perera O, Balas-Nakash M, Schiffman-Selechnik E, Barbato-Dosal A, Vadillo-Ortega F. 2007. Obesity increases metabolic syndrome risk factors in school-aged children from an urban school in Mexico city. *J Am Diet Assoc* 107:81-91.
- Pignoli P, Tremoli E, Poli A, Oreste P, Paoletti R. 1986. Intimal plus media thickness of the arterial wall: a direct measurement with ultrasound imaging. *Circulation* 74:1399-1406.
- Pi J, Kumagai Y, Sun G, Yamauchi H, Yoshida T, Iso H, et al. 2000. Decreased serum concentrations of nitric oxide metabolites among Chinese in an endemic area of chronic arsenic poisoning in inner Mongolia. *Free Radic Biol Med* 28:1137-1142.
- Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. 1998. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. *Lancet* 351:88-92.
- Roman DA, Pizarro I, Rivera L, Camara C, Palacios MA, Gomez MM, et al. 2011. An approach to the arsenic status in cardiovascular tissues of patients with coronary heart disease. *Hum Exp Toxicol* 30:1150-1164.
- Rosenberg HG. 1974. Systemic arterial disease and chronic arsenicism in infants. *Arch Pathol* 97:360-365.
- Simeonova PP, Hulderman T, Harki D, Luster MI. 2003. Arsenic exposure accelerates atherogenesis in apolipoprotein E(-/-) mice. *Environ Health Perspect* 111:1744-1748.
- Simeonova PP, Luster MI. 2004. Arsenic and atherosclerosis. *Toxicol Appl Pharmacol* 198:444-449.
- Slyper AH. 2004. Clinical review 168: What vascular ultrasound testing has revealed about pediatric atherogenesis, and a potential clinical role for ultrasound in pediatric risk assessment. *J Clin Endocrinol Metab* 89:3089-3095.

- Srivastava S, D'Souza SE, Sen U, States JC. 2007. In utero arsenic exposure induces early onset of atherosclerosis in ApoE^{-/-} mice. *Reprod Toxicol* 23:449-456.
- States JC, Srivastava S, Chen Y, Barchowsky A. 2009. Arsenic and cardiovascular disease. *Toxicol Sci* 107:312-323.
- Suda O, Tsutsui M, Morishita T, Tasaki H, Ueno S, Nakata S, et al. 2004. Asymmetric dimethylarginine produces vascular lesions in endothelial nitric oxide synthase-deficient mice: involvement of renin-angiotensin system and oxidative stress. *Arterioscler Thromb Vasc Biol* 24:1682-1688.
- Tain YL, Huang LT. 2011. Asymmetric dimethylarginine: clinical applications in pediatric medicine. *J Formos Med Assoc* 110:70-77.
- Wang CH, Jeng JS, Yip PK, Chen CL, Hsu LI, Hsueh YM, et al. 2002. Biological gradient between long-term arsenic exposure and carotid atherosclerosis. *Circulation* 105:1804-1809.
- Wang CH, Hsiao CK, Chen CL, Hsu LI, Chiou HY, Chen SY, et al. 2007. A review of the epidemiologic literature on the role of environmental arsenic exposure and cardiovascular diseases. *Toxicol Appl Pharmacol* 222:315-326.
- Wu MM, Chiou HY, Hsueh YM, Hong CT, Su CL, Chang SF, et al. 2006. Effect of plasma homocysteine level and urinary monomethylarsonic acid on the risk of arsenic-associated carotid atherosclerosis. *Toxicol Appl Pharmacol* 216:168-175.
- Zarazua S, Rios R, Delgado JM, Santoyo ME, Ortiz-Perez D, Jimenez-Capdeville ME. 2010. Decreased arginine methylation and myelin alterations in arsenic exposed rats. *Neurotoxicology* 31:94-100.

Table 1. Child characteristics, urinary arsenicals levels, plasmatic biomarkers and cIMT of study population in Zimapan, Mexico.

Variable		n	Percentage or GM ^a (range)	Mean±SD
Sex	Male	107	54%	
	Female	92	46%	
In Utero exposure	Yes	164	82%	
	No	35	18%	
Age (years)	≤5 years old	140	70%	5.26±1.49
	>5 years old	59	30%	
BMI (kg/m ²)		195	16.02 (11.8-26)	16.17±2.31
BMI z-score (percentile)		195	40.01 (1-99)	55.27±30.68
Urinary Arsenic (ng/mL)	iAs	195	5.41 (0.57-100.78)	8.70±11.61
	MAs		5.41 (0.21-55.67)	7.98±8.31
	DMAs		46.69 (4.93-236.95)	57.27±40.22
	tAs		59.14 (5.71-369.94)	74.31±57.04
Urinary Arsenic (%)	iAs	195	9.15 (2.33-72.08)	10.27±6.21
	MAs		9.14(1.50-17.98)	9.67±3.07
	DMAs		78.95 (24.77-91.52)	79.59±8.51
Plasma Analysis		193		
Glucose (mg/dL)			83 (61-130)	83±11
Total cholesterol (mg/dL)			147 (13-284)	150±29
Triglycerides (mg/dL)			73 (20-333)	81±42
HDL cholesterol (mg/dL)			51 (30-103)	52±12
LDL cholesterol (mg/dL)			79 (20-168)	82±22
VLDL cholesterol (mg/dL)			15 (4-67)	16±8
Atherogenic index			2.86 (1.2-5.3)	2.92±0.58
Hemoglobin (g/dL)		194	13.86 (11.7-17.1)	13.89±0.87
Hematocrit (%)		194	39.78 (33.5-48.7)	39.84±2.32
Leucocytes (number/mL)		194	6.35 (3.4-12.8)	6.52±1.56
Cardiovascular Risk Biomarkers				
ADMA (μmol/L)		196	0.69(0.23-1.43)	0.72±0.19
sVCAM-1 (μg/mL)		177	1.50 (0.68-4.56)	1.56±0.50
sICAM-1 (μg /mL)		196	0.60 (0.33-1.42)	0.62±0.15
Carotid Ultrasonography (mm)		152		
cIMTmin			0.32 (0.08-0.54)	0.33±0.08
cIMTmean			0.43 (0.24-0.8)	0.44±0.07
cIMTmax			0.54 (0.28-1.28)	0.55±0.13

^aGM-Geometric mean

Table 2. Relation of children characteristics, lipid serum profile, plasma adhesion molecules and urinary arsenic exposure with plasma ADMA or cIMTmin

Variable	ADMA (μmol/L)			cIMT(mm)		
	β Coef.	95% CI	p	β Coef.	95% CI	p
Age (years)	-0.017	-0.034, 0.0007	0.061	0.005	-0.005, 0.016	0.290
Gender	0.041	-0.012, 0.093	0.130	0.010	-0.016, 0.036	0.451
BMI kg/m ²	0.0055	-0.006, 0.017	0.353	0.006	0.0004, 0.012	0.037
BMI zscore (percentile)	0.0006	-0.0002, 0.002	0.147	0.0006	0.0002, 0.0010	0.005
BMI categories						
Underweight	0.011	-0.096, 0.119	0.836	-0.023	-0.073, 0.026	0.348
Overweight	0.049	-0.021, 0.12	0.168	0.029	-0.005, 0.063	0.090
Obesity	0.029	-0.064, 0.12	0.54	0.011	-0.035, 0.057	0.633
Plasma Analyses						
Triglycerides(mg/dL)	0.0007	0.00002, 0.0013	0.043	0.0002	-0.000082, 0.0005	0.146
VLDL cholesterol(mg/dL)	0.0034	0.00007, 0.007	0.046	0.0012	-0.00041, 0.0027	0.147
Atherogenic index	0.0047	-0.041, 0.051	0.84	0.024	0.0017, 0.046	0.035
Adhesion Molecules						
sICAM-1 (μg/mL)	0.00024	0.000065, 0.0004	0.007	0.00008	-5.16e-6, 0.00017	0.065
sVCAM-1 (μg/mL)	0.0001	0.000045, 0.00015	0.000	0.00002	-3.55e-6, 4.7e-5	0.092
tAs Categories (ng/mL)						
35-70	-0.017	-0.09, 0.055	0.640	0.033	-0.0004, 0.067	0.053
>70	0.010	-0.064, 0.085	0.785	0.054	0.019, 0.089	0.003
Urinary As (ng/mL)						
tAs	0.0004	-0.00007, 0.0009	0.092	0.0002	7.48e-6, 0.0005	0.043
iAs	0.0012	-0.0011, 0.004	0.306	0.0009	-0.0002, 0.002	0.124
MAs	0.0028	-0.0005, 0.006	0.093	0.0013	-0.0003, 0.003	0.101
DMAs	0.0006	-0.00006, 0.0013	0.076	0.0003	0.00003, 0.0007	0.034

Males were compared with females. Body mass index categories were compared with normal weight category. tAs categories were compared with <35ng/mL category. Simple linear regression analyses were used to compare untransformed ADMA or cIMTmin with continuous variables or categorical data.

Table 3. Robust multivariable linear regression analysis of associations between cIMTmin and arsenic levels and cardiovascular markers in children (R-squared =0.18, P=0.0000, N=141).

Explanatory Variable	β Coefficient^a	95% CI	P Value
tAs in urine (35-70 ng/mL) ^b	0.035	-0.0028, 0.072	0.070
tAs in urine (>70 ng/mL) ^b	0.058	0.0198, 0.095	0.003
Plasmatic ADMA (μ mol/L)	0.068	0.0117, 0.124	0.018
Atherogenic index	0.019	-0.0007, 0.038	0.059
z-score (percentile)	0.0005	0.00007, 0.0009	0.023
Age (years)	0.008	-0.0011, 0.016	0.088

^a β coefficient represents the average difference in cIMT (mm) per unit change in the explanatory variable.

^bSum of inorganic and methylated arsenic species.

Table 4. Multivariable linear regression analysis of associations between plasma ADMA ($\mu\text{mol/L}$) in children and explanatory variables. (R-squared =0.19; Adj R-squared=0.151; P=0.0002; N= 128).

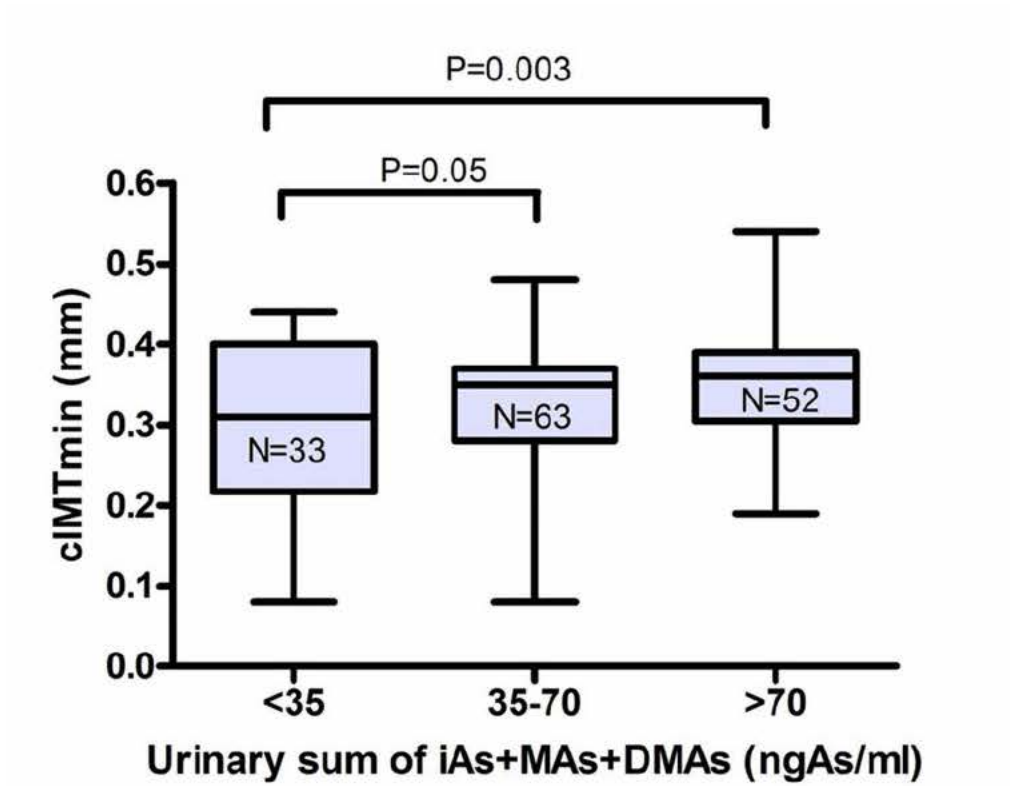
Explanatory Variable	β Coefficient ^a	95% CI	P Value
iAs (%)	0.0147	0.003, 0.026	0.014
DMAs (%)	0.006	-0.0009, 0.0129	0.086
sVCAM-1 ($\mu\text{g/mL}$)	0.000086	0.00002, 0.00015	0.008
Triglycerides (mg/dL)	0.00067	-0.0002, 0.0016	0.145
cIMTmin (mm)	0.4189	0.0010, 0.837	0.049
Age (years)	-0.0314	-0.056, -0.0069	0.012

^a β coefficient represents the average difference in ADMA in $\mu\text{mol/L}$ per unit change in the explanatory variable.

Figure Legends

Figure 1. Box-plot graphs for carotid intima media thickness by level of sum of inorganic and methylated arsenic species in urine of children. The outer bounds of the boxes represent the interquartile range; the median is represented by the midline. The whiskers represent the adjacent values. Analysis of Variance was performed to assess the cIMT increase over tAs categories. Additionally, Wilcoxon type test for trend across urinary arsenic categories were evaluated (P=0.027).

Figure 2. Predictors of plasma ADMA ($\mu\text{mol/L}$) in children according to multivariable linear regression. A) Urinary iAs%, B) urinary DMAs%, C) plasma sVCAM-1, D) plasma triglycerides, E) carotid intima-media thickness (min), and F) age.



+ Figure 1
85x68mm (300 x 300 DPI)

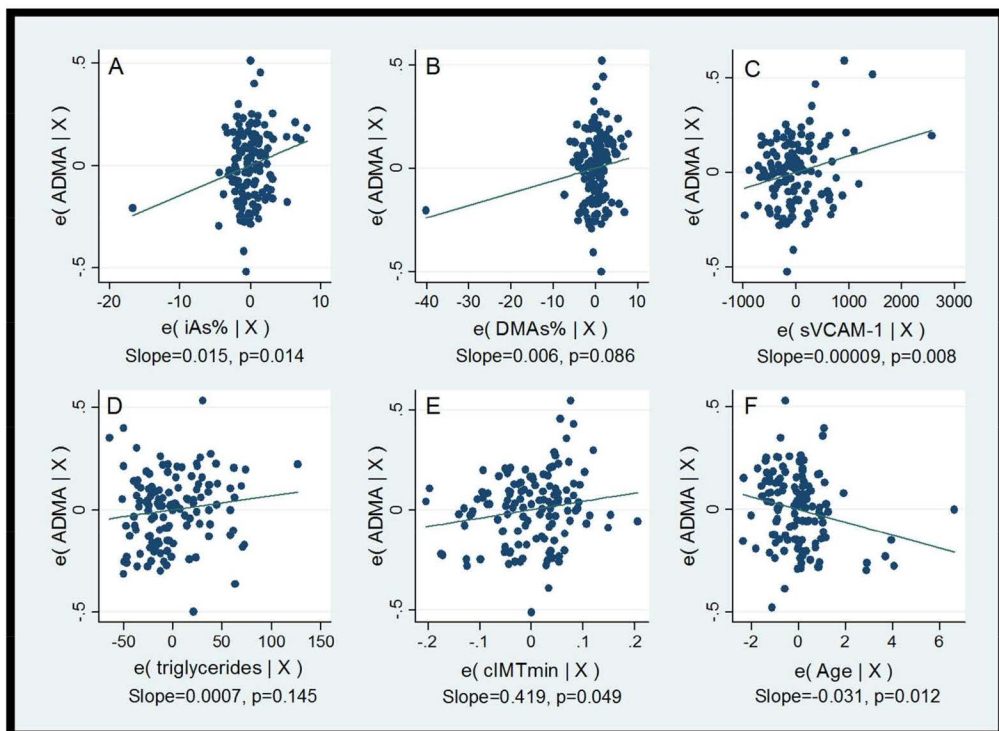


Figure 2

251x184mm (150 x 150 DPI)